

**Title:** **OOMYCETE-RESISTANT TRANSGENIC  
PLANTS BY VIRTUE OF PATHOGEN-  
INDUCED EXPRESSION OF A  
HETEROLOGOUS HYPERSENSITIVE  
RESPONSE ELICITOR**

**Inventors:** **Steven V. Beer and  
David W. Bauer**

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**OOMYCETE-RESISTANT TRANSGENIC PLANTS BY VIRTUE OF  
PATHOGEN-INDUCED EXPRESSION OF A HETEROLOGOUS  
HYPERSENSITIVE RESPONSE ELICITOR**

5           This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/178,565, filed January 26, 2000, which is hereby incorporated by reference in its entirety.

10          This invention was made in part with support by the U.S. Government under Grant No. 97-34367-3937 from the U.S. Department of Agriculture. The U.S. Government may have certain rights in this invention.

**FIELD OF THE INVENTION**

15          The present invention relates to transgenic plants resistant to oomycete infection which contain a heterologous hypersensitive response elicitor under the control of a promoter responsive to infection by an oomycete.

**BACKGROUND OF THE INVENTION**

20          In general, fungal plant diseases can be classified into two types: those caused by soilborne fungi and those caused by airborne fungi. Soilborne fungi cause some of the most widespread and serious plant diseases, such as root and stem rot caused by *Fusarium spp.* and root rot caused by *Phytophthora spp.* For example, *Phytophthora parasitica* var. *nicotiana*, a soilborne oomycete found in many tobacco growing regions worldwide, causes black shank, a highly destructive root and stem rot disease of many varieties of cultivated tobacco.

25          Since airborne fungi can be spread long distances by wind, they can cause devastating losses, particularly in crops which are grown over large regions. A number of pathogens have caused widespread epidemics in a variety of crops.  
30          Important diseases caused by airborne fungi are stem rust (*Puccinia graminis*) on wheat, corn smut (*Ustilago maydis*) on corn, and late blight disease (*Phytophthora infestans*) on potato and tomato. *Plasmopera viticola* is an airborne oomycete that causes downy mildew disease on grape vines. The blue mold fungus (*Peronospora*

*tabacina*) has caused catastrophic losses in tobacco crops, particularly in the United States and Cuba.

Most of these fungal diseases are difficult to combat, and farmers and growers must use a combination of practices, such as sanitary measures, resistant 5 cultivars, and effective fungicide against such diseases. Hundreds of millions of dollars are spent annually for chemical control of plant-pathogenic fungi. As a result, there is today a real need for new, more effective and safe means to control plant-pathogenic fungi, particularly oomycetes which are responsible for major crop loss.

Genetic engineering promises to be an effective strategy for reducing 10 the losses associated with diseases of field crops. Several successful approaches have been reported where the constitutive expression of antimicrobial peptides such as cecropins (Arce et al., "Enhanced Resistance to Bacterial Infection by Erwinia Carotovora Susp. Atroseptica in Transgenic Potato Plants Expressing the Attacin or the Cecropin SB-37 Genes," Am. J. Potato Res. 76:169-177 (1999)), lysozyme 15 (Nakajima et al., "Fungal and Bacterial Disease Resistance in Transgenic Plants Expressing Human Lysozyme," Plant Cell Reports 16:674-679 (1997)), and monoclonal antibodies (Tavladoraki et al, "Transgenic Plants Expressing a Functional Single Chain FV Antibody are Specifically Protected from Virus Attack," Nature 366:468-472 (1993)) effectively protected plants from parasitic organisms. However 20 successful, these approaches have limited application to food production since many of these antimicrobial peptides and plant defense molecules are potentially toxic or allergenic to humans (Franck-Oberaspach et al., "Consequences of Classical and Biotechnological Resistance Breeding for Food Toxicology and Allergenicity," Plant Breeding 116:1-17 (1997)). Thus, alternative approaches for genetically engineering 25 disease resistance would be more desirable.

Plants posses a highly evolved pathogen surveillance system which allows for recognition of specific pathogen derived molecules known as elicitors. Elicitor recognition results in an incompatible plant-microbe interaction, defined as the rapid activation of plant defense genes, typically resulting in the hypersensitive 30 response and the onset of systemic acquired resistance.

The hypersensitive response is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly, Z.,

“Defenses Triggered by the Invader: Hypersensitivity,” pages 201-224 in: Plant Disease: An Advanced Treatise, Vol. 5, J.G. Horsfall and E.B. Cowling, ed. Academic Press New York (1980); Klement, Z., “Hypersensitivity,” pages 149-177 in: Phytopathogenic Prokaryotes, Vol. 2, M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The hypersensitive response elicited by bacteria is readily observed as a tissue collapse if high concentrations ( $\geq 10^7$  cells/ml) of a limited host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into the leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement, Z., “Rapid Detection of Pathogenicity of Phytopathogenic Pseudomonads,” Nature 199:299-300; Klement, et al., “Hypersensitive Reaction Induced by Phytopathogenic Bacteria in the Tobacco Leaf,” Phytopathology 54:474-477 (1963); Turner, et al., “The Quantitative Relation Between Plant and Bacterial Cells Involved in the Hypersensitive Reaction,” Phytopathology 64:885-890 (1974); Klement, Z., “Hypersensitivity,” pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York (1982)). The capacities to elicit the hypersensitive response in a nonhost and be pathogenic in a host appear linked. As noted by Klement, Z., “Hypersensitivity,” pages 149-177 in Phytopathogenic Prokaryotes, Vol. 2., M.S. Mount and G.H. Lacy, ed. Academic Press, New York, (1982), these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the hypersensitive response or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren, P.B., et al., “Gene Cluster of *Pseudomonas syringae* pv. ‘phaseolicola’ Controls Pathogenicity of Bean Plants and Hypersensitivity on Nonhost Plants,” J. Bacteriol. 168:512-22 (1986); Willis, D.K., et al., “*hrp* Genes of Phytopathogenic Bacteria,” Mol. Plant-Microbe Interact. 4:132-138 (1991)). Consequently, the hypersensitive response may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in Gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis, D.K., et al., “*hrp* Genes of Phytopathogenic Bacteria,” Mol. Plant-Microbe Interact. 4:132-138 (1991); Bonas, U., “*hrp* Genes of Phytopathogenic Bacteria,” pages 79-98 in: Current Topics in Microbiology and Immunology: Bacterial Pathogenesis of Plants

and Animals - Molecular and Cellular Mechanisms, J.L. Dangl, ed. Springer-Verlag, Berlin (1994)). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem, et al., "Evolutionary Conservation of Pathogenicity Determinants Among Plant and Animal Pathogenic Bacteria," *Trends Microbiol.* 1:175-180 (1993)). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich, protein elicitors of the hypersensitive response (He, S.Y., et al. "Pseudomonas Syringae pv. Syringae Harpin<sub>PSS</sub>: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255-1266 (1993); Wei, Z.-M., et al., "HrpI of *Erwinia amylovora* Functions in Secretion of Harpin and is a Member of a New Protein Family," *J. Bacteriol.* 175:7958-7967 (1993); Arlat, M., et al. "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of Pseudomonas solanacearum," *EMBO J.* 13:543-553 (1994)).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei, Z.-M., et al, "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," *Science* 257:85-88 (1992)). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit a hypersensitive response in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 *PopA1* protein has similar physical properties and also elicits the hypersensitive response in leaves of tobacco, which is not a host of that strain (Arlat, et al., "PopA1, a Protein Which Induces a Hypersensitive-like Response on Specific Petunia Genotypes, is Secreted via the Hrp Pathway of Pseudomonas solanacearum," *EMBO J.* 13:543-53 (1994)). However, *P. solanacearum* *popA* mutants still elicit the hypersensitive response in tobacco and incite disease in tomato. Thus, the role of these glycine-rich hypersensitive response elicitors can vary widely among Gram-negative plant pathogens.

Other plant pathogenic hypersensitive response elicitors have been isolated, cloned, and sequenced. These include: *Erwinia chrysanthemi* (Bauer, et. al., "Erwinia chrysanthemi Harpin<sub>Ech</sub>: Soft-Rot Pathogenesis," *MPMI* 8(4): 484-91

(1995)); *Erwinia carotovora* (Cui, et. al., "The RsmA<sup>-</sup> Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrpN*<sub>Ecc</sub> and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1966)); *Erwinia stewartii* (Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," 8th Int'l. Cong. Molec. Plant-Microb. Inter. July 14-19, 1996 and Ahmad, et. al., "Harpin is not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," Ann. Mtg. Am. Phytopath. Soc. July 27-31, 1996); and *Pseudomonas syringae* pv. *syringae* (WO 94/26782 to Cornell Research Foundation, Inc.).

Because the hypersensitive response results in localized necrosis of plant tissue, it is desirable to limit expression of a heterologous hypersensitive response elicitor to certain tissues in transgenic plants. This approach is discussed generally in PCT publication WO 94/01546 to Beer et al., but no specific transgenic plants are identified and only two suitable fungus-responsive promoters are suggested, e.g., the phenylalanine ammonia lyase and chalcone synthase promoters. No promoters responsive specifically to infection by oomycetes are identified therein.

The present invention is directed to overcoming these and other deficiencies in the art.

## SUMMARY OF THE INVENTION

The present invention relates to a chimeric gene that includes a first DNA molecule encoding a hypersensitive response elicitor protein or polypeptide, a promoter operably linked 5' to the first DNA molecule to induce transcription of the first DNA molecule in response to activation of the promoter by an oomycete, and a 25 3' regulatory region operably linked to the first DNA molecule. Also disclosed are an expression system that includes a vector in which is inserted a chimeric gene of the present invention and a host cell that includes a chimeric gene of the present invention.

Another aspect of the present invention relates to a transgenic plant 30 resistant to disease resulting from oomycete infection. The transgenic plant includes a chimeric gene of the present invention, wherein the promoter induces transcription of the first DNA molecule in response to infection of the plant by an oomycete.

Transgenic seeds and transgenic cultivars obtained from the transgenic plant are also disclosed.

An additional aspect of the present invention relates to a method of making a recombinant plant cell. This is accomplished by transforming a plant cell  
5 with a chimeric gene of the present invention under conditions effective to yield transcription of the first DNA molecule in response to oomycete-induced activation of the promoter.

A further aspect of the present invention relates to a method of making a plant resistant to disease resulting from oomycete infection. This is accomplished  
10 by transforming a plant cell with a chimeric gene of the present invention under conditions effective to yield transcription of the first DNA molecule in response to oomycete-induced activation of the promoter and regenerating the plant from the transformed plant cell.

The present invention confers oomycete-induced disease resistance to plants transformed with a chimeric gene encoding a hypersensitive response elicitor protein or polypeptide, which is transcribed within a limited population of plant cells in response to infection of the plant by an oomycete. To limit transcription of the chimeric gene within a certain population of plant cells, the chimeric gene includes a promoter that is responsive to infection by an oomycete (i.e., it is activated by the oomycete). The hypersensitive response elicitor protein or polypeptide can cause tissue collapse at the site of infection and/or induce systemic resistance against the oomycete and other pathogens. By using the promoter from the potato *gst1* gene, for example, which is activated by infection with oomyceteous fungi, the present invention can control fungal pathogens within crops without harming the transgenic plant and without resorting to use of environmentally damaging chemicals.  
25

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation and partial restriction map of T-DNA in plant transformation vector pCPP1294. Filled triangles represent the left and right borders; *Pgst1* represents the *gst1* promoter from potato variety Atlantic; PR1-b represents the DNA molecule encoding a signal sequence from *Nicotiana tabacum*;  
30

*hrpN* represents the DNA molecule encoding the hypersensitive response elicitor harpin<sub>Ea</sub> of *Erwinia amylovora*; NT represents the nos terminating region; *aacC1* represents the gentamycin resistance cassette.

Figure 2 is an image of transgenic *Arabidopsis* plants containing a construct encoding GUS under control of the *gst1* promoter. To demonstrate pathogen inducibility of the *gst1* promoter in *Arabidopsis*, GUS staining was measured following inoculation of the plants with water (left) or *P. parasitica* (right). GUS expression is indicated by dark staining.

Figures 3A and 3B show an analysis of *hrpN* gene expression in *Arabidopsis* transgenic line GSSN8-4, containing the construct shown in Figure 1, after inoculation with *P. parasitica* NOCO. At one day intervals leaves were collected for isolation of total RNA. Figure 3A is a Northern blot analysis performed using *hrpN* DNA as a probe. Figure 3B is an ethidium bromide stained gel shown as a control (bottom).

Figures 4A and 4B are images demonstrating *Arabidopsis* GSSN 8-4 are resistant to *P. parasitica*. Figure 4A shows the effects of *P. parasitica* infection in WT Arabidopsis (control, left) and GSSN 8-4 Arabidopsis (test, right). Figure 4B shows the degree of trypan blue staining of *P. parasitica*-infected leaves of WT (control, left) and GSSN 8-4 plants (test, right), both taken 10 days post-inoculation.

Figure 5 is a graph depicting the severity of *P. parasitica* infection in WT (control), EV (control), and *hrpN* transgenic plants (test). Two week old plants were drop inoculated with conidiospores of *P. parasitica* (2 ml drops;  $5 \times 10^4$  spores/ml). Ten days after inoculation, 30 plants of each genotype were rated for disease severity. Ratings were adapted from Cao et al. ("Generation of Broad-Spectrum Disease Resistance by Overexpression of an Essential Regulatory Gene in Systemic Acquired Resistance," *Proc. Natl. Acad. Sci. USA* 95:6531-6536 (1998), which is hereby incorporated by reference) as follows: 1, no conidiophores present on plant; 2, 0-5 conidiophores per infected plant; 3, 6-20 conidiophores present on a few infected leaves; 4, 6-20 conidiophores present on most infected leaves; 5, more than 20 conidiophores on all infected leaves.

## DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention relates to a novel DNA construct in the form of a chimeric gene. The chimeric gene includes a first DNA molecule 5 encoding a hypersensitive response elicitor protein or polypeptide, a promoter operably linked 5' to the first DNA molecule to induce transcription of the first DNA molecule in response to activation of the promoter by an oomycete, and a 3' regulatory region operably linked to the first DNA molecule. As discussed more fully hereinafter, a chimeric gene of the present invention is particularly useful in preparing 10 a transgenic plant for the purpose of rendering the transgenic plant resistant to disease resulting from infection thereof by an oomycete.

The first DNA molecule can encode any hypersensitive response elicitor protein or polypeptide which is effective in triggering a hypersensitive response (i.e., in a particular host plant selected for transformation). Generally, it is 15 desirable to express hypersensitive response elicitors only in plants which are non-hosts for the source organism of the hypersensitive response elicitor. Suitable hypersensitive elicitor proteins or polypeptides are those derived from a wide variety of bacterial and fungal pathogens, preferably bacterial pathogens.

Exemplary hypersensitive response elicitor proteins and polypeptides 20 from bacterial sources include, without limitation, the hypersensitive response elicitors from *Erwinia* species (e.g., *Erwinia amylovora*, *Erwinia chrysanthemi*, *Erwinia stewartii*, *Erwinia carotovora*, etc.), *Pseudomonas* species (e.g., *Pseudomonas syringae*, *Pseudomonas solanacearum*, etc.), and *Xanthomonas* species (e.g., *Xanthomonas campestris*). In addition to hypersensitive response elicitors from 25 these Gram-negative bacteria, it is possible to use elicitors from Gram-positive bacteria. One example is the hypersensitive response elicitor from *Clavibacter michiganensis* subsp. *sepedonicus*.

Exemplary hypersensitive response elicitor proteins or polypeptides 30 from fungal sources include, without limitation, the hypersensitive response elicitors (i.e., elicins) from various *Phytophthora* species (e.g., *Phytophthora parasitica*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora capsici*, *Phytophthora megasperma*, *Phytophthora citrophthora*, etc.).

Preferably, the first DNA molecule encodes a hypersensitive response elicitor protein or polypeptide of *Erwinia chrysanthemi*, *Erwinia amylovora*, *Pseudomonas syringae*, or *Pseudomonas solanacearum*.

The hypersensitive response elicitor protein or polypeptide from

- 5    *Erwinia chrysanthemi* has an amino acid sequence corresponding to SEQ. ID. No. 1 as follows:

	Met Gln Ile Thr Ile Lys Ala His Ile Gly Gly Asp Leu Gly Val Ser	
	1                        5                        10                        15	
10	Gly Leu Gly Ala Gln Gly Leu Lys Gly Leu Asn Ser Ala Ala Ser Ser	
	20                        25                        30	
	Leu Gly Ser Ser Val Asp Lys Leu Ser Ser Thr Ile Asp Lys Leu Thr	
	35                        40                        45	
15	Ser Ala Leu Thr Ser Met Met Phe Gly Gly Ala Leu Ala Gln Gly Leu	
	50                        55                        60	
	Gly Ala Ser Ser Lys Gly Leu Gly Met Ser Asn Gln Leu Gly Gln Ser	
	65                        70                        75                        80	
	Phe Gly Asn Gly Ala Gln Gly Ala Ser Asn Leu Leu Ser Val Pro Lys	
	85                        90                        95	
20	Ser Gly Gly Asp Ala Leu Ser Lys Met Phe Asp Lys Ala Leu Asp Asp	
	100                      105                      110	
	Leu Leu Gly His Asp Thr Val Thr Lys Leu Thr Asn Gln Ser Asn Gln	
	115                      120                      125	
25	Leu Ala Asn Ser Met Leu Asn Ala Ser Gln Met Thr Gln Gly Asn Met	
	130                      135                      140	
	Asn Ala Phe Gly Ser Gly Val Asn Asn Ala Leu Ser Ser Ile Leu Gly	
	145                      150                      155                      160	
	Asn Gly Leu Gly Gln Ser Met Ser Gly Phe Ser Gln Pro Ser Leu Gly	
	165                      170                      175	
30	Ala Gly Gly Leu Gln Gly Leu Ser Gly Ala Gly Ala Phe Asn Gln Leu	
	180                      185                      190	
	Gly Asn Ala Ile Gly Met Gly Val Gly Gln Asn Ala Ala Leu Ser Ala	
	195                      200                      205	
35	Leu Ser Asn Val Ser Thr His Val Asp Gly Asn Asn Arg His Phe Val	
	210                      215                      220	
	Asp Lys Glu Asp Arg Gly Met Ala Lys Glu Ile Gly Gln Phe Met Asp	
	225                      230                      235                      240	

Gln Tyr Pro Glu Ile Phe Gly Lys Pro Glu Tyr Gln Lys Asp Gly Trp  
245 250 255

Ser Ser Pro Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser Lys  
260 265 270

5 Pro Asp Asp Asp Gly Met Thr Gly Ala Ser Met Asp Lys Phe Arg Gln  
275 280 285

Ala Met Gly Met Ile Lys Ser Ala Val Ala Gly Asp Thr Gly Asn Thr  
290 295 300

10 Asn Leu Asn Leu Arg Gly Ala Gly Gly Ala Ser Leu Gly Ile Asp Ala  
305 310 315 320

Ala Val Val Gly Asp Lys Ile Ala Asn Met Ser Leu Gly Lys Leu Ala  
325 330 335

Asn Ala

15 This hypersensitive response elicitor protein or polypeptide has a molecular weight of 34 kDa, is heat stable, has a glycine content of greater than 16%, and contains substantially no cysteine. This *Erwinia chrysanthemi* hypersensitive response elicitor protein or polypeptide is encoded by a DNA molecule having a nucleotide sequence  
20 corresponding to SEQ. ID. No. 2 as follows:

cgattttacc cgggtgaacg tgctatgacc gacagcatca cggtattcga caccgttacg 60  
gcgtttatgg ccgcgatgaa ccggcatcag gcggcgcgct ggtcgccgca atccggcgtc 120  
gatctggtat ttcaagttgg ggacacccggg cgtgaactca ttagtgcagat tcagccgggg 180  
25 cagcaatatac ccggcatgtt ggcgcacgctg ctcgctcgtc gtatcagca ggcggcagag 240  
tgcgatggct gccatctgtg cctgaacggc agcgatgtat tgatcctctg gtggcccgctg 300  
ccgtcggatc ccggcaggtta tccgcagggtg atcgaacgtt tggtaact ggcgggaatg 360  
acgttgcgt cgctatccat agcaccgacg ggcgcgtccgc agacaggaa cggacgcgcc 420  
cgatcattaa gataaaggcg gctttttta ttgcaaaaacg gtaacggtga ggaaccgttt 480  
30 caccgtcggc gtcactcagt aacaagtatac catcatgatg cctacatcgg gatcggcgctg 540  
ggcatccgtt gcagatactt ttgcgaacac ctgacatgaa tgaggaaacg aaattatgca 600  
aattacgatc aaagcgcaca tcggcggtga tttggcgctc tccggctctgg ggctgggtgc 660  
tcagggactg aaaggactga attccgcggc ttcatcgctg ggttccagcg tggataaaact 720  
gagcagcacc atcgataagt tgacctccgc gctgacttcg atgatgtttg gcccgcgcgt 780  
35 ggccgcagggg ctggcgcca gctcgaaggg gctggggatg agcaatcaac tggccacagtc 840  
tttcggcaat ggccgcgcagg gtgcgagcaa cctgctatcc gtaccgaaat ccggccggcga 900

5           tgcgttgtca aaaatgtttg ataaagcgt ggacgatctg ctgggtcatg acaccgtac 960  
caagctgact aaccagagca accaactggc taattcaatg ctgaacgcc a gccagatgac 1020  
ccagggtaat atgaatgcgt tcggcagcgg tgtgaacaac gcactgtcgt ccattctcg 1080  
caacggtctc ggccagtcga tgagtggctt ctctcagcct tctctgggg caggcggctt 1140  
5           gcagggcctg agcggcgcgg gtgcattcaa ccagttgggt aatgccatcg gcatggcgt 1200  
ggggcagaat gctgcgtga gtgcgttgag taacgtcagc acccacgtag acgtaacaa 1260  
ccgcccacttt gtagataaag aagatcgcgg catggcgaaa gagatcggcc agtttatgga 1320  
tcagtatcog gaaatattcg gtaaaaccgga ataccagaaa gatggctgga gttcgccgaa 1380  
gacggacgac aaatcctggg ctaaagcgt gagtaaaccg gatgatgacg gatgaccgg 1440  
10          cgccagcatg gacaaattcc gtcaggcgat gggtatgatc aaaagcgcgg tggcgggtga 1500  
taccggcaat accaacctga acctgcgtgg cgccggcggt gcatcgctgg gtatcgatgc 1560  
ggctgtcgtc ggcgataaaa tagccaacat gtcgtgggt aagctggcca acgcctgata 1620  
atctgtgctg gcctgataaaa gcggaaacga aaaaagagac ggggaaggct gtctctttc 1680  
ttattatgcg gtttatgcgg ttacctggac cggttaatca tcgtcatcg a tctggtacaa 1740  
15          acgcacattt tcccgttcat tcgcgtcggt acgcgcacaca atcgatgg catcttcctc 1800  
gtcgctcaga ttgcgcggct gatggggAAC gccgggtggatataagagaa actcgccggc 1860  
cagatggaga cacgtctgcg ataaatctgt gccgtaacgt gtttctatcc gcccctttag 1920  
cagatagatt gcggtttcgat aatcaacatg gtaatgcggt tccgcctgtg cgccggccgg 1980  
gatcaccaca atattcatag aaagctgtct tgcacctacc gatcgccgg agataccgac 2040  
20          aaaatagggc agttttcgat tggtatccgt ggggtgttcc ggcctgacaa tcttgagttg 2100  
gttcgtcatac atctttctcc atctggcgat cctgatcggt t 2141

The hypersensitive response elicitor protein or polypeptide derived from *Erwinia amylovora* has an amino acid sequence corresponding to SEQ. ID.

25 No. 3 as follows:

	Met Ser Leu Asn Thr Ser Gly Leu Gly Ala Ser Thr Met Gln Ile Ser
	1                       5                       10                       15
30	Ile Gly Gly Ala Gly Gly Asn Asn Gly Leu Leu Gly Thr Ser Arg Gln
	20                       25                       30
	Asn Ala Gly Leu Gly Gly Asn Ser Ala Leu Gly Leu Gly Gly Asn
	35                       40                       45
	Gln Asn Asp Thr Val Asn Gln Leu Ala Gly Leu Leu Thr Gly Met Met
	50                       55                       60

Met Met Met Ser Met Met Gly Gly Gly Leu Met Gly Gly Gly Leu  
65 70 75 80

Gly Gly Gly Leu Gly Asn Gly Leu Gly Gly Ser Gly Gly Leu Gly Glu  
85 90 95

5 Gly Leu Ser Asn Ala Leu Asn Asp Met Leu Gly Gly Ser Leu Asn Thr  
100 105 110

Leu Gly Ser Lys Gly Gly Asn Asn Thr Thr Ser Thr Thr Asn Ser Pro  
115 120 125

10 Leu Asp Gln Ala Leu Gly Ile Asn Ser Thr Ser Gln Asn Asp Asp Ser  
130 135 140

Thr Ser Gly Thr Asp Ser Thr Ser Asp Ser Ser Asp Pro Met Gln Gln  
145 150 155 160

Leu Leu Lys Met Phe Ser Glu Ile Met Gln Ser Leu Phe Gly Asp Gly  
165 170 175

15 Gln Asp Gly Thr Gln Gly Ser Ser Ser Gly Gly Lys Gln Pro Thr Glu  
180 185 190

Gly Glu Gln Asn Ala Tyr Lys Lys Gly Val Thr Asp Ala Leu Ser Gly  
195 200 205

20 Leu Met Gly Asn Gly Leu Ser Gln Leu Leu Gly Asn Gly Gly Leu Gly  
210 215 220

Gly Gly Gln Gly Gly Asn Ala Gly Thr Gly Leu Asp Gly Ser Ser Leu  
225 230 235 240

Gly Gly Lys Gly Leu Gln Asn Leu Ser Gly Pro Val Asp Tyr Gln Gln  
245 250 255

25 Leu Gly Asn Ala Val Gly Thr Gly Ile Gly Met Lys Ala Gly Ile Gln  
260 265 270

Ala Leu Asn Asp Ile Gly Thr His Arg His Ser Ser Thr Arg Ser Phe  
275 280 285

30 Val Asn Lys Gly Asp Arg Ala Met Ala Lys Glu Ile Gly Gln Phe Met  
290 295 300

Asp Gln Tyr Pro Glu Val Phe Gly Lys Pro Gln Tyr Gln Lys Gly Pro  
305 310 315 320

Gly Gln Glu Val Lys Thr Asp Asp Lys Ser Trp Ala Lys Ala Leu Ser  
325 330 335

35 Lys Pro Asp Asp Asp Gly Met Thr Pro Ala Ser Met Glu Gln Phe Asn  
340 345 350

Lys Ala Lys Gly Met Ile Lys Arg Pro Met Ala Gly Asp Thr Gly Asn  
355 360 365

Gly Asn Leu Gln Ala Arg Gly Ala Gly Gly Ser Ser Leu Gly Ile Asp  
370 375 380

Ala Met Met Ala Gly Asp Ala Ile Asn Asn Met Ala Leu Gly Lys Leu  
385 390 395 400

5 Gly Ala Ala

This hypersensitive response elicitor protein or polypeptide has a molecular weight of about 39 kDa, has a pI of approximately 4.3, and is heat stable at 100°C for at least 10 minutes. This hypersensitive response elicitor protein or polypeptide has substantially no cysteine. The hypersensitive response elicitor protein or polypeptide derived from *Erwinia amylovora* is more fully described in Wei, Z-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference. The DNA molecule encoding this hypersensitive response elicitor protein or polypeptide has a 15 nucleotide sequence corresponding to SEQ. ID. No. 4 as follows:

aagcttcggc atggcacgtt tgaccgttgg gtcggcaggg tacgtttgaa ttattcataa 60  
gaggaatacg ttatgagtct gaataacaagt gggctggag cgtcaacgtat gcaaatttct 120  
atccggcggtg cgggcggaaa taacgggttg ctgggtacca gtcgccagaa tgctgggttg 180  
20 ggtggcaatt ctgcactggg gctgggcggc ggtaatcaaa atgataaccgt caatcagctg 240  
gctggcttac tcaccggcat gatgatgatg atgagcatga tggcggtgg tgggctgatg 300  
ggcggtggct taggcgggtgg cttaggtaat ggcttgggtg gctcaggtgg cctggcgaa 360  
ggactgtcga acgcgctgaa cgatatgtta ggccggttcgc tgaacacgct gggctcgaaa 420  
ggccggcaaca ataccacttc aacaacaaat tccccgctgg accaggcgct gggtattaac 480  
25 tcaacgtccc aaaacgacga ttccacctcc ggcacagatt ccacctcaga ctccagcgcac 540  
ccgatgcagc agctgctgaa gatgttcago gagataatgc aaagcctgtt tggtgatggg 600  
caagatggca cccagggcag ttccctctggg ggcaagcagc cgaccgaagg cgagcagaac 660  
gcctataaaaa aaggagtcac tggatgcgtg tcgggcctga tgggtaatgg tctgagccag 720  
ctccttggca acggggact gggaggtggt cagggcggta atgctggcac gggtcttgcac 780  
30 ggttcgtcgc tgggcggcaa agggctgcaa aacctgagcg ggccgggtggc ctaccagcag 840  
ttaggtaacg ccgtgggtac cggtatcggt atgaaagcgg gcattcaggc gctgaatgat 900  
atcggtacgc acaggcacag ttcaaccgt tctttcgtca ataaaggcga tcgggcgtatg 960  
gcgaaggaaa tcggtcagtt catggaccag tatcctgagg tgggtggcaa gccgcgtac 1020  
cagaaaggcc cgggtcagga ggtgaaaacc gatgacaaat catggcataa agcactgagc 1080

aagccagatg acgacggaat gacaccagcc agtatggagc agttcaacaa agccaagggc 1140  
atgatcaaaa ggcccattggc gggtgatacc ggcaacggca acctgcaggc acgcggtgcc 1200  
ggtggttctt cgctgggtat tgatgccatg atggccggtg atgccattaa caatatggca 1260  
cttggcaagc tggcgccggc ttaagctt 1288

5

The hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas syringae* has an amino acid sequence corresponding to SEQ. ID. No. 5 as follows:

10 Met Gln Ser Leu Ser Leu Asn Ser Ser Ser Leu Gln Thr Pro Ala Met  
1 5 10 15  
Ala Leu Val Leu Val Arg Pro Glu Ala Glu Thr Thr Gly Ser Thr Ser  
20 25 30  
15 Ser Lys Ala Leu Gln Glu Val Val Val Lys Leu Ala Glu Glu Leu Met  
35 40 45  
Arg Asn Gly Gln Leu Asp Asp Ser Ser Pro Leu Gly Lys Leu Leu Ala  
50 55 60  
20 Lys Ser Met Ala Ala Asp Gly Lys Ala Gly Gly Ile Glu Asp Val  
65 70 75 80  
Ile Ala Ala Leu Asp Lys Leu Ile His Glu Lys Leu Gly Asp Asn Phe  
85 90 95  
Gly Ala Ser Ala Asp Ser Ala Ser Gly Thr Gly Gln Gln Asp Leu Met  
100 105 110  
25 Thr Gln Val Leu Asn Gly Leu Ala Lys Ser Met Leu Asp Asp Leu Leu  
115 120 125  
Thr Lys Gln Asp Gly Gly Thr Ser Phe Ser Glu Asp Asp Met Pro Met  
130 135 140  
30 Leu Asn Lys Ile Ala Gln Phe Met Asp Asp Asn Pro Ala Gln Phe Pro  
145 150 155 160  
Lys Pro Asp Ser Gly Ser Trp Val Asn Glu Leu Lys Glu Asp Asn Phe  
165 170 175  
Leu Asp Gly Asp Glu Thr Ala Ala Phe Arg Ser Ala Leu Asp Ile Ile  
180 185 190  
35 Gly Gln Gln Leu Gly Asn Gln Gln Ser Asp Ala Gly Ser Leu Ala Gly  
195 200 205  
Thr Gly Gly Gly Leu Gly Thr Pro Ser Ser Phe Ser Asn Asn Ser Ser  
210 215 220

Val Met Gly Asp Pro Leu Ile Asp Ala Asn Thr Gly Pro Gly Asp Ser  
225 230 235 240

Gly Asn Thr Arg Gly Glu Ala Gly Gln Leu Ile Gly Glu Leu Ile Asp  
245 250 255

5 Arg Gly Leu Gln Ser Val Leu Ala Gly Gly Gly Leu Gly Thr Pro Val  
260 265 270

Asn Thr Pro Gln Thr Gly Thr Ser Ala Asn Gly Gly Gln Ser Ala Gln  
275 280 285

10 Asp Leu Asp Gln Leu Leu Gly Gly Leu Leu Leu Lys Gly Leu Glu Ala  
290 295 300

Thr Leu Lys Asp Ala Gly Gln Thr Gly Thr Asp Val Gln Ser Ser Ala  
305 310 315 320

Ala Gln Ile Ala Thr Leu Leu Val Ser Thr Leu Leu Gln Gly Thr Arg  
325 330 335

15 Asn Gln Ala Ala Ala  
340

This hypersensitive response elicitor protein or polypeptide has a molecular weight of 34-35 kDa. It is rich in glycine (about 13.5%) and lacks cysteine and tyrosine.

20 Further information about the hypersensitive response elicitor derived from *Pseudomonas syringae* is found in He, S. Y., et al., "Pseudomonas syringae pv. syringae Harpin<sub>Pss</sub>: a Protein that is Secreted via the Hrp Pathway and Elicits the Hypersensitive Response in Plants," *Cell* 73:1255-1266 (1993), which is hereby incorporated by reference. The DNA molecule encoding this hypersensitive response elicitor from *Pseudomonas syringae* has a nucleotide sequence corresponding to SEQ. ID. No. 6 as follows:

atgcagagtc tcagtcttaa cagcagctcg ctgcaaacc cggcaatggc ccttgcctg 60  
gtacgtcctg aagccgagac gactggcagt acgtcgagca aggcgcattca ggaagttgtc 120  
30 gtgaagctgg ccgaggaact gatgcgcaat ggtcaactcg acgacagctc gccattggaa 180  
aaactgttgg ccaagtcgat ggccgcagat ggcaaggcgg gcccggat tggatgtc 240  
atcgctgcgc tggacaagct gatccatgaa aagctcggtg acaacttcgg cgcgtctgcg 300  
gacagcgcct cgggtaccgg acagcaggac ctgatgactc aggtgctcaa tggcctggcc 360  
aagtcgatgc tcgatgatct tctgaccaag caggatggcg ggacaagctt ctccgaagac 420  
35 gatatgccga tgctgaacaa gatcgccgac ttcatggatg acaatcccgc acagttccc 480  
aagccggact cgggctcctg ggtgaacgaa ctcaaggaag acaacttcct tcatggcgac 540

gaaacggctg cgttccgttc ggcaactcgac atcattggcc agcaactggg taatcagcag 600  
agtgacgctg gcagtcggc agggacgggt ggaggtctgg gcactccgag cagttttcc 660  
aacaactcgt ccgtgatggg tgatccgctg atcgacgcca ataccggtcc cggtgacagc 720  
ggcaataccc gtggtgaagc gggcaactg atcggcgagc ttatcgaccg tggcctgcaa 780  
5 tcggtattgg cccgtggtgg actgggcaca cccgtaaaca ccccgccagac cggtaacgtcg 840  
gcgaatggcg gacagtccgc tcaggatctt gatcagttgc tggcggctt gctgctcaag 900  
ggctggagg caacgctcaa ggatgccggg caaacaggca ccgacgtgca gtcgagcgct 960  
gcgcaaatcg ccaccttgct ggtcagtagc ctgctgcaag gcacccgcaa tcaggctgca 1020  
gcctga 1026

10

Another potentially suitable hypersensitive response elicitor from *Pseudomonas syringae* is disclosed in U.S. Patent Application Serial No. 09/120,817, which is hereby incorporated by reference.

15 The hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas solanacearum* has an amino acid sequence corresponding to SEQ. ID. No. 7 as follows:

	Met	Ser	Val	Gly	Asn	Ile	Gln	Ser	Pro	Ser	Asn	Leu	Pro	Gly	Leu	Gln
	1					5				10					15	
20	Asn	Leu	Asn	Leu	Asn	Thr	Asn	Thr	Asn	Ser	Gln	Gln	Ser	Gly	Gln	Ser
						20			25					30		
	Val	Gln	Asp	Leu	Ile	Lys	Gln	Val	Glu	Lys	Asp	Ile	Leu	Asn	Ile	Ile
						35			40					45		
25	Ala	Ala	Leu	Val	Gln	Lys	Ala	Ala	Gln	Ser	Ala	Gly	Gly	Asn	Thr	Gly
						50			55					60		
	Asn	Thr	Gly	Asn	Ala	Pro	Ala	Lys	Asp	Gly	Asn	Ala	Asn	Ala	Gly	Ala
						65			70			75		80		
	Asn	Asp	Pro	Ser	Lys	Asn	Asp	Pro	Ser	Lys	Ser	Gln	Ala	Pro	Gln	Ser
						85			90					95		
30	Ala	Asn	Lys	Thr	Gly	Asn	Val	Asp	Asp	Ala	Asn	Asn	Gln	Asp	Pro	Met
						100			105					110		
	Gln	Ala	Leu	Met	Gln	Leu	Leu	Glu	Asp	Leu	Val	Lys	Leu	Leu	Lys	Ala
						115			120					125		
35	Ala	Leu	His	Met	Gln	Gln	Pro	Gly	Gly	Asn	Asp	Lys	Gly	Asn	Gly	Val
						130			135			140				
	Gly	Gly	Ala	Asn	Gly	Ala	Lys	Gly	Ala	Gly	Gly	Gln	Gly	Gly	Leu	Ala
						145			150			155		160		

Glu Ala Leu Gln Glu Ile Glu Gln Ile Leu Ala Gln Leu Gly Gly Gly  
165 170 175

Gly Ala Gly Ala Gly Gly Ala Gly Gly Gly Val Gly Gly Ala Gly Gly  
180 185 190

5 Ala Asp Gly Gly Ser Gly Ala Gly Gly Ala Gly Gly Ala Asn Gly Ala  
195 200 205

Asp Gly Gly Asn Gly Val Asn Gly Asn Gln Ala Asn Gly Pro Gln Asn  
210 215 220

10 Ala Gly Asp Val Asn Gly Ala Asn Gly Ala Asp Asp Gly Ser Glu Asp  
225 230 235 240

Gln Gly Gly Leu Thr Gly Val Leu Gln Lys Leu Met Lys Ile Leu Asn  
245 250 255

Ala Leu Val Gln Met Met Gln Gln Gly Gly Leu Gly Gly Asn Gln  
260 265 270

15 Ala Gln Gly Gly Ser Lys Gly Ala Gly Asn Ala Ser Pro Ala Ser Gly  
275 280 285

Ala Asn Pro Gly Ala Asn Gln Pro Gly Ser Ala Asp Asp Gln Ser Ser  
290 295 300

20 Gly Gln Asn Asn Leu Gln Ser Gln Ile Met Asp Val Val Lys Glu Val  
305 310 315 320

Val Gln Ile Leu Gln Gln Met Leu Ala Ala Gln Asn Gly Gly Ser Gln  
325 330 335

Gln Ser Thr Ser Thr Gln Pro Met  
340

25

Further information regarding this hypersensitive response elicitor protein or polypeptide derived from *Pseudomonas solanacearum* is set forth in Arlat, M., et al., "PopA1, a Protein which Induces a Hypersensitive-like Response in Specific Petunia Genotypes, is Secreted via the Hrp Pathway of *Pseudomonas solanacearum*," EMBO J. 13:543-533 (1994), which is hereby incorporated by reference. It is encoded by a DNA molecule from *Pseudomonas solanacearum* having a nucleotide sequence corresponding SEQ. ID. No. 8 as follows:

atgtcagtcg gaaacatcca gagccgtcg aacctcccgg gtctgcagaa cctgaacctc 60  
35 aacaccaaca ccaacagcca gcaatcgggc cagtcgtgc aagacctgat caaggcaggc 120  
gagaaggaca tcctcaacat catgcagcc ctctgtcaga aggccgcaca gtcggcgggc 180  
ggcaacaccg gtaacaccgg caacgcgcgg gcgaaggacg gcaatgccaa cgccggcgcc 240

aacgaccgcga gcaagaacga cccgagcaag agccaggctc cgcatcgcc caacaagacc 300  
ggcaacgtcg acgacgc当地 caaccaggat ccgatgcaag cgctgatgca gctgctggaa 360  
gacctggta agctgctgaa ggccgc当地 cacatgc当地 agccccggc当地 caatgacaag 420  
ggcaacggcg tggcggtgc caacggcgcc aagggtgccc gcccgc当地 cggcctggcc 480  
5 gaagcgctgc aggagatcga gcagatactc gcccagctcg gcccggc当地 tgctggcgcc 540  
ggccggcgcc ggccgggtgt cggcggtgct ggtggcgcc当地 atggcggtc当地 cggtgccgg 600  
ggccgc当地 ggccggcg gtgc当地 acggcc当地 ggccatggcg tgaacggcaa ccaggc当地 ac 660  
ggcccgccaga acgc当地 acggcga tgtcaacggt gccaacggcg cggatgacgg cagc当地 ac 720  
caggc当地 cggcc tcaccggcgt gctgcaaaag ctgatgaaga tcctgaacgc gctggc当地 cag 780  
10 atgatgc当地 aaggc当地 cccct cggccggc当地 aaccaggc当地 agggc当地 cccct gaagggtgcc 840  
ggcaacgc当地 cccggcttc cggcgcaac ccggccgca accagcccg ttc当地 cggat 900  
gatcaatcg cccggccaga caatctgcaa tcccgatca tggatgtggt gaaggaggc 960  
gtccagatcc tgc当地 cagat gctggcgcc cagaacggcg gcagccagca gtccacctcg 1020  
acgc当地 cggcga tgtaa 1035

15

Other embodiments of the present invention include, but are not limited to, use of the nucleotide sequence encoding for the hypersensitive response elicitor protein or polypeptide from *Erwinia carotovora* and *Erwinia stewartii*.

Isolation of *Erwinia carotovora* hypersensitive response elicitor protein or polypeptide is described in Cui, et al., "The RsmA Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp N<sub>Ecc</sub>* and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," *MPMI*, 9(7):565-73 (1996), which is hereby incorporated by reference. The hypersensitive response elicitor protein or polypeptide of *Erwinia stewartii* is set forth in Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," *8th Int'l. Cong. Molec. Plant-Microbe Interact.*, July 14-19, 1996 and Ahmad, et al., "Harpin is Not Necessary for the Pathogenicity of *Erwinia stewartii* on Maize," *Ann. Mtg. Am. Phytopath. Soc.*, July 27-31, 1996, which are hereby incorporated by reference.

The hypersensitive response elicitor proteins or polypeptides from various *Phytophthora* species are described in Kaman, et al., "Extracellular Protein Elicitors from Phytophthora: Most Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens," *Molec. Plant-Microbe Interact.*, 6(1):15-25 (1993); Ricci, et al., "Structure and Activity of Proteins from Pathogenic Fungi

Phytophthora Eliciting Necrosis and Acquired Resistance in Tobacco," Eur. J. Biochem., 183:555-63 (1989); Ricci, et al., "Differential Production of Parasiticein, and Elicitor of Necrosis and Resistance in Tobacco, by Isolates of Phytophthora parasitica," Plant Path. 41:298-307 (1992); Baillreul, et al., "A New Elicitor of the 5 Hypersensitive Response in Tobacco: A Fungal Glycoprotein Elicits Cell Death, Expression of Defense Genes, Production of Salicylic Acid, and Induction of Systemic Acquired Resistance," Plant J., 8(4):551-60 (1995), and Bonnet, et al., "Acquired Resistance Triggered by Elicitors in Tobacco and Other Plants," Eur. J. Plant Path., 102:181-92 (1996), which are hereby incorporated by reference.

10 Another hypersensitive response elicitor in accordance with the present invention is from *Clavibacter michiganensis* subsp. *sepedonicus* which is described in U.S. Patent Application Serial No. 09/136,625, which is hereby incorporated by reference.

15 Other elicitors can be readily identified by isolating putative hypersensitive response elicitors and testing them for elicitor activity as described, for example, in Wei, Z-M., et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (1992), which is hereby incorporated by reference. Cell-free preparations from culture supernatants can be tested for elicitor activity (i.e., local necrosis) by using them to 20 infiltrate appropriate plant tissues. Once identified, DNA molecules encoding a hypersensitive response elicitor can be isolated using standard techniques known to those skilled in the art. The isolated DNA molecule can then be introduced into the chimeric gene for expression in a transgenic plant of the present invention.

The first DNA molecule can also encode fragments of the above 25 hypersensitive response elicitor proteins or polypeptides as well as fragments of full length elicitors from other pathogens.

Suitable fragments can be produced by several means. Subclones of the gene encoding a known elicitor protein can be produced using conventional molecular genetic manipulation for subcloning gene fragments, such as described by 30 Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), and Ausubel et al. (ed.), Current Protocols in Molecular Biology, John Wiley & Sons (New York, NY) (1999 and preceding

editions), which are hereby incorporated by reference. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller protein or polypeptide that can be tested for elicitor activity, e.g., using procedures set forth in Wei, Z-M., et al., Science 257: 85-88 (1992), which is hereby incorporated by reference.

- 5 In another approach, based on knowledge of the primary structure of the protein, fragments of the elicitor protein gene may be synthesized using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. Erlich, H.A., et al., "Recent Advances in the Polymerase Chain Reaction," Science 252:1643-51 (1991), which is hereby incorporated by reference.
- 10 These can then be cloned into an appropriate vector for expression of a truncated protein or polypeptide from bacterial cells as described above.

- An example of suitable fragments of a hypersensitive response elicitor which elicit a hypersensitive response are fragments of the *Erwinia amylovora* hypersensitive response elicitor protein or polypeptide of SEQ. ID. No. 3. The  
15 fragments can be a C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, an N-terminal fragment of the amino acid sequence of SEQ. ID. No. 3, or an internal fragment of the amino acid sequence of SEQ. ID. No. 3. The C-terminal fragment of the amino acid sequence of SEQ. ID. No. 3 can span amino acids 105 and 403 of SEQ. ID. No. 3. The N-terminal fragment of the amino acid sequence of SEQ.  
20 ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 1 and 98, 1 and 104, 1 and 122, 1 and 168, 1 and 218, 1 and 266, 1 and 342, 1 and 321, and 1 and 372. The internal fragment of the amino acid sequence of SEQ. ID. No. 3 can span the following amino acids of SEQ. ID. No. 3: 76 and 209, 105 and 209, 99 and 209, 137 and 204, 137 and 200, 109 and 204, 109 and 200, 137 and 180, and 105 and 180.  
25 DNA molecules encoding these fragments can also be utilized in the chimeric gene of the present invention.

- The first DNA molecule also can be a DNA molecule that hybridizes under stringent conditions to the DNA molecule having nucleotide sequence of SEQ. ID. Nos. 2, 4, 6, or 8. An example of suitable stringency conditions is when  
30 hybridization is carried out at a temperature of about 37°C using a hybridization medium that includes 0.9M sodium citrate ("SSC") buffer, followed by washing with 0.2x SSC buffer at 37°C. Higher stringency can readily be attained by increasing the

temperature for either hybridization or washing conditions or increasing the sodium concentration of the hybridization or wash medium. Nonspecific binding may also be controlled using any one of a number of known techniques such as, for example, blocking the membrane with protein-containing solutions, addition of heterologous

- 5 RNA, DNA, and SDS to the hybridization buffer, and treatment with RNase. Wash conditions are typically performed at or below stringency. Exemplary high stringency conditions include carrying out hybridization at a temperature of about 42°C to about 65°C for up to about 20 hours in a hybridization medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.2%  
10 ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 50 µg/ml *E. coli* DNA, followed by washing carried out at between about 42°C to about 65°C in a 0.2x SSC buffer.

Variants of suitable hypersensitive response elicitor proteins or polypeptides can also be expressed by the first DNA molecule. Variants may be made by, for example, the deletion, addition, or alteration of amino acids that have minimal influence on the properties, secondary structure and hydrophobic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide (such as a 6xHis tag).

The promoter of the chimeric gene should be selected on the basis of its ability to induce transcription of the first DNA molecule in response to infection of the plant by an oomycete (i.e., the oomycete activates the promoter).

- 25 According to one embodiment, the promoter preferably includes some or all of the promoter-effective regions of a *gst1* gene from potato. The *gst1* promoter is activated in response to infection by oomycetes and not by wounding or other environmental perturbations. The *gst1* promoter from potato has a nucleic acid sequence corresponding to SEQ. ID. No. 9 as follows:

30  
gaattcagga agaattttgt aggttcaact aaattatata tatatatata aaaaaataaa 60  
aattattaga cgcttcgact atttacttac tttaaaattt gaattttcgta acgaataaaa 120  
ttatgtca gagaaaagtc ttttagctat tcacatgcta ggaagttca cttttgggtgg 180

atcagtgatt	gtatattatt	taatatata	caatttctc	atcaaactga	aatgaaaaga	240
taaaattaat	ataaaaaact	ccattcattt	taatttattt	tcatgtttt	acttgatcca	300
aaatctaaca	atttaaaagg	ttttaaattt	tttgtcctt	ttttaaatta	aaaatatgtc	360
aaatatatta	aaatataattt	tttaaatttt	atactaaaaa	acatgtcaca	tgaatattt	420
5	aaattataaa	attatcaaaa	ataaaaaaaag	aatatttctt	taacaaatta	aaattgaaaa
	tatgataaat	aaattaaact	attctatcat	tgattttct	agccaccaga	540
	cagtgggtga	catgagcaca	taagtcatct	ttattgtatt	tttattactca	ctccaaaaat
	atagggata	tgtttactac	ttaatttagt	caaataata	tttatattag	aataattgaa
	tagtcaaaca	agaaaacttta	atgcatcctt	attttt		696

10

Effective fragments of SEQ. ID. No. 9 are also encompassed by the present invention. U.S. Patent Nos. 5,750,874 and 5,723,760 to Strittmayer et al., which are hereby incorporated by reference, define promoter-effective regions of the potato *gst1* promoter. Preferably, the *gst1* promoter includes a nucleotide sequence corresponding, at a minimum, to nucleotides 295-567 of SEQ. ID. No. 9. The *gst1* promoter can also include effective portions containing nucleotides 295-696 of SEQ. ID. No. 9.

The chimeric gene of the present invention also includes an operable 3' regulatory region, selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA for expression in plant cells, operably linked to the first DNA molecule which encodes for a hypersensitive response elicitor. A number of 3' regulatory regions are known to be operable in plants. Exemplary 3' regulatory regions include, without limitation, the nopaline synthase 3' regulatory region (Fraley, et al., "Expression of Bacterial Genes in Plant Cells," *Proc. Nat'l Acad. Sci. USA*, 80:4803-4807 (1983), which is hereby incorporated by reference) and the cauliflower mosaic virus 3' regulatory region (Odell, et al., "Identification of DNA Sequences Required for Activity of the Cauliflower Mosaic Virus 35S Promoter," *Nature*, 313(6005):810-812 (1985), which is hereby incorporated by reference). Virtually any 3' regulatory region known to be operable in plants would suffice for proper expression of the coding sequence of the chimeric gene of the present invention.

The first DNA molecule, promoter, and a 3' regulatory region can be ligated together using well known molecular cloning techniques described in

Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press, NY (1989), which is hereby incorporated by reference.

The chimeric gene can also include a second DNA molecule encoding a secretion signal. A number of suitable secretion signals are known in the art and other are continually being identified. The secretion signal can be an RNA leader which directs secretion of the subsequently transcribed protein or polypeptide, or the secretion signal can be an amino terminal peptide sequence that is recognized by a host plant secretory pathway. The second DNA molecule can be ligated between the promoter and the first DNA molecule, using known molecular cloning techniques as indicated above.

According to one embodiment, the second DNA molecule encodes a secretion signal derived from *Nicotiana tabacum*. Specifically, this DNA molecule encodes the secretion signal polypeptide for *PR1-b* gene of *Nicotiana tabacum*. This second DNA molecule has a nucleotide sequence corresponding to SEQ. ID. No. 10 as follows:

tctagaccat gggattttt ctctttcac aaatgccctc atttttctt gtgtcgacac 60  
ttctcttatt cctaataata tctcaactttt ctcatgccca aaactctaga 110  
20 The above sequence includes XbaI sites (underlined) at each end to facilitate insertion  
of the second DNA molecule into the chimeric gene of the present invention. The  
coding sequence of SEQ. ID. No. 10 starts at base 8. The polypeptide encoded by this  
nucleic acid molecule has an amino acid sequence corresponding to SEQ. ID. No. 11  
25 as follows:

An alternative second DNA molecule encoding the secretion signal polypeptide for *PR1-b* gene of *Nicotiana tabacum* has a nucleotide sequence corresponding to SEQ. ID. No. 12 as follows:

atgggatttt ttctctttc acaaatgcc tcatttttc ttgtctctac acttctctta 60  
ttcctaataa tatctcaactc ttctcatgcc caaaaactctc aa 102

- 5 This nucleotide sequence is disclosed in Genbank Accession No. X03465, which is hereby incorporated by reference. The polypeptide encoded by this nucleic acid molecule has an amino acid sequence corresponding to SEQ. ID. No. 13 as follows:

10 Met Gly Phe Phe Leu Phe Ser Gln Met Pro Ser Phe Phe Leu Val Ser  
1 5 10 15  
Thr Leu Leu Leu Phe Leu Ile Ile Ser His Ser Ser His Ala Gln Asn  
20 25 30  
Ser Gln

- 15 Yet another second DNA molecule encodes the secretion signal for the *PR1-a* gene of *Nicotiana tabacum*. This DNA molecule has a nucleotide sequence corresponding to SEQ. ID. No. 14 as follows:

20 atgggatttg ttctctttc acaattgcct tcatttttc ttgtctctac acttctctta 60  
ttcctagtaa tatcccaactc ttgccgtgcc 90

- 25 This DNA molecule is disclosed in Genbank Accession No. X06361, which is hereby incorporated by reference. The polypeptide encoded by this nucleic acid molecule has an amino acid sequence corresponding to SEQ. ID. No. 15 as follows:

30 Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val Ser  
1 5 10 15  
Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala  
20 25 30

- Still another second DNA molecule encodes the secretion signal for the *PR4-a* gene of *Nicotiana tabacum*. This DNA molecule has a nucleotide sequence corresponding to SEQ. ID. No. 16 as follows:

40 atggagagag ttaataatta taagttgtgc gtggcattgt tgatcatcag catggtgatg 60  
gcaatggcg 75  
cgcca

This DNA molecule is disclosed in Genbank Accession No. X58546, which is hereby incorporated by reference. The polypeptide encoded by this nucleic acid molecule has an amino acid sequence corresponding to SEQ. ID. No. 17 as follows:

5 Met Glu Arg Val Asn Asn Tyr Lys Leu Cys Val Ala Leu Leu Ile Ile  
 1 5 10 15  
 Ser Met Val Met Ala Met Ala Ala Ala  
 20 25

10 Each second DNA molecule can be cloned using primers that introduce restriction sites at the 5' and 3' ends thereof to facilitate insertion of the second DNA molecule into the chimeric gene of the present invention. SEQ. ID. No. 10 is shown to include such restriction sites (e.g., XbaI).

Further aspects of the present invention include an expression system  
15 that includes a vector containing a chimeric gene of the present invention, as well as a host cell which includes a chimeric gene of the present invention. As described more fully hereinafter, the recombinant host cell can be either a bacterial cell (i.e., *Agrobacterium*) or a plant cell. In the case of recombinant plant cells, it is preferable that the chimeric gene is stably inserted into the genome of the recombinant plant cell.

20 The chimeric gene can be incorporated into cells using conventional recombinant DNA technology. Generally, this involves inserting the chimeric gene into an expression vector or system to which it is heterologous (i.e., not normally present). As described above, the chimeric gene contains the necessary elements for the transcription and translation in plant cells of the first DNA molecule (i.e.,  
25 encoding the hypersensitive response elicitor protein or polypeptide) and, if present, the second DNA molecule.

U.S. Patent No. 4,237,224 issued to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Once the chimeric gene of the present invention has been prepared, it is ready to be incorporated into a host cell. Recombinant molecules can be introduced

into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Springs Laboratory, Cold Springs Harbor, 5 New York (1989), which is hereby incorporated by reference. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like. Preferably the host cells are either a bacterial cell or a plant cell.

Accordingly, another aspect of the present invention relates to a method of making a recombinant plant cell. Basically, this method is carried out by 10 transforming a plant cell with a chimeric gene of the present invention under conditions effective to yield transcription of the first DNA molecule in response to oomycete-induced activation of the promoter. Preferably, the chimeric gene is stably inserted into the genome of the recombinant plant cell as a result of the transformation.

15 A related aspect of the present invention concerns a method of making a plant resistant to disease resulting from oomycete infection. Basically, this method is carried out by transforming a plant cell with a chimeric gene of the present invention under conditions effective to yield transcription of the first DNA molecule in response to oomycete-induced activation of the promoter and regenerating a plant 20 from the transformed plant cell.

One approach to transforming plant cells with a chimeric gene of the present invention is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in 25 U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford, et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles 30 with the vector containing the heterologous DNA. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector

and heterologous DNA) can also be propelled into plant cells. Other variations of particle bombardment, now known or hereafter developed, can also be used.

Another method of introducing the chimeric gene is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies that contain the chimeric gene. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The chimeric gene may also be introduced into the plant cells by electroporation. Fromm, et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are 10 electroporated in the presence of plasmids containing the chimeric gene. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the chimeric gene into plant cells is to 15 infect a plant cell with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* previously transformed with the chimeric gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for 48 to 72 hours on 20 regeneration medium without antibiotics at 25-28°C.

*Agrobacterium* is a representative genus of the Gram-negative family Rhizobiaceae. Its species are responsible for crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes*). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized 25 only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences such as a chimeric gene of the present invention can be introduced into appropriate plant cells by means of the Ti plasmid of 30 *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into

the plant genome. Schell, J., Science, 237:1176-83 (1987), which is hereby incorporated by reference.

Plant tissue suitable for transformation include leaf tissue, root tissue, meristems, zygotic and somatic embryos, and anthers.

5 After transformation, the transformed plant cells can be selected and regenerated.

Preferably, transformed cells are first identified using, e.g., a selection marker simultaneously introduced into the host cells along with the chimeric gene of the present invention. Suitable selection markers include, without limitation, markers 10 coding for antibiotic resistance, such as kanamycin resistance (Fraley, et al., Proc. Natl. Acad. Sci. USA, 80:4803-4807 (1983), which is hereby incorporated by reference). A number of antibiotic-resistance markers are known in the art and other are continually being identified. Any known antibiotic-resistance marker can be used 15 to transform and select transformed host cells in accordance with the present invention. Cells or tissues are grown on a selection media containing an antibiotic, whereby generally only those transformants expressing the antibiotic resistance marker continue to grow.

Once a recombinant plant cell or tissue has been obtained, it is possible 20 to regenerate a full-grown plant therefrom. Thus, another aspect of the present invention relates to a transgenic plant that is resistant to disease resulting from oomycete infection. The transgenic plant includes a chimeric gene of the present invention, wherein the promoter induces transcription of the first DNA molecule in response to infection of the plant by an oomycete. Preferably, the chimeric gene is stably inserted into the genome of the transgenic plant of the present invention.

25 Plant regeneration from cultured protoplasts is described in Evans, et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

30 It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of rice, wheat, barley, rye, cotton, sunflower, peanut, corn, potato, sweet potato, bean, pea, chicory, lettuce,

endive, cabbage, cauliflower, broccoli, turnip, radish, spinach, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, melon, strawberry, grape, raspberry, pineapple, soybean, tobacco, tomato, sorghum, and sugarcane.

5 Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing transformed explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form  
10 plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and  
15 repeatable.

After the chimeric gene is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing or by preparing cultivars. With respect to sexual crossing, any of a number of standard breeding techniques can be used depending upon the species to be crossed. Cultivars can be propagated in accord  
20 with common agricultural procedures known to those in the field.

Resistance against different types of oomycetes may be imparted to transgenic plants according to the present invention. Without being bound by any particular theory, it is believed that a hypersensitive response elicitor protein or polypeptide encoded by the first DNA molecule is transcribed in response to infection  
25 of the plant by an oomycete. The exact mechanism by which the promoter is activated to regulate transcription of sequences under its control is not fully understood; however, the first DNA molecule is transcribed and the hypersensitive response elicitor is expressed in a limited population of cells (i.e., those in which transcription has been induced following oomycete infection). Once expressed, it is  
30 believed that the hypersensitive response elicitor can either be secreted from the plant cell (assuming the chimeric gene also contains a second DNA molecule encoding an N-terminal secretion signal) or leaked from an oomycete-infected plant cell.

Regardless of how the hypersensitive response elicitor is delivered to the intercellular environment, it is believed that the hypersensitive response elicitor protein or polypeptide will initiate a hypersensitive response to cause localized necrosis of oomycete-infected tissues. In addition, systemic acquired resistance may be

5 developed by the transgenic plant following initiation of the hypersensitive response. This may yield broad disease and/or pathogen resistance to the transgenic plants of the present invention.

Oomycetes against which resistance is imparted include, without limitation, species of *Plasmopara*, *Phytophthora*, *Peronospora*, *Pseudoperonospora*,  
10 *Bremia*, *Sclerospora*, *Aphanomyces*, *Pythium*, and *Albugo*.

According to one embodiment of the present invention, an oomycete resistant transgenic tobacco plant includes a chimeric gene of the present invention, wherein expression of the encoded hypersensitive response elicitor is responsive to infection of the plant by an oomycete that is a pathogen of tobacco, including, but not  
15 limited to, *Peronospora tabacina* (which causes blue mold) and *Phyophthora parasitica* (which causes black shank).

The chimeric gene of the present invention can be utilized to impart oomycete resistance for a wide variety of tobacco plants, some of which may possess varying levels of natural resistance against pathogenic oomycetes. The varieties of tobacco plants which can be protected include, without limitation, those referred to as Coker 371 Gold, K 149, K 326, K 346, K 394, K 730, RG 11, RG17, RG22, Speight G-70, Speight G-117, Speight G-126, GL939, NC 55, NC 71, NC 72, NC 95, NC  
20 2326, OX 207, OX 940, RG 81, RG H4, RG H61, Speight 168, Speight NF3, Speight 172, CU 236, CU 387, CU 368, NC TG91, OX 4142NF, OX 4083, RG 4H2-12, RG  
25 4H2-17, RG 4H2-20, Speight 177, Speight 178, Speight 179, VPI 107, VPI 605, NG TG94, KY 14, KY 8959, KY 907, KY 908, TN 86, TN 90, TN 97, VA 116, VA 509, B 21 x KY 10, KY 14 x L8, NC 3, NC BH129, DH332, COOP 313, COOP 543, Clay's 403, Clay's 502, HY 402, PF 561, and R 711.

According to another embodiment of the present invention, an  
30 oomycete resistant transgenic grape plant includes a chimeric gene of the present invention, wherein expression of the encoded hypersensitive response elicitor is responsive to infection of the plant by an oomycete that is a pathogen of grape,

including, but not limited to, *Plasmopara viticola* (which causes downy mildew), *Pythium* spp. (which cause root and/or stem rot), and *Phytophthora* spp. (which cause root and/or stem rot).

The chimeric gene of the present invention can be utilized to impart  
5 oomycete resistance for a wide variety of grapevine plants. The chimeric gene is particularly well suited to imparting resistance to *Vitis* scion or rootstock cultivars. Scion cultivars which can be protected include, without limitation, those commonly referred to as Table or Raisin Grapes, such as Alden, Almeria, Anab-E-Shahi, Autumn Black, Beauty Seedless, Black Cornish, Black Damascus, Black Malvoisie,  
10 Black Prince, Blackrose, Bronx Seedless, Burgrave, Calmeria, Campbell Early, Canner, Cardinal, Catawba, Christmas, Concord, Dattier, Delight, Diamond, Dizmar, Duchess, Early Muscat, Emerald Seedless, Emperor, Exotic, Ferdinand de Lesseps, Fiesta, Flame seedless, Flame Tokay, Gasconade, Gold, Himrod, Hunisa, Hussiene, Isabella, Italia, July Muscat, Khandahar, Katta, Kourgane, Kishmishi, Loose Perlette,  
15 Malaga, Monukka, Muscat of Alexandria, Muscat Flame, Muscat Hamburg, New York Muscat, Niabell, Niagara, Olivette blanche, Ontario, Pierce, Queen, Red Malaga, Ribier, Rish Baba, Romulus, Ruby Seedless, Schuyler, Seneca, Suavis (IP 365), Thompson seedless, and Thomuscatt. They also include, without limitation, those used in wine production, such as Aleatico, Alicante Bouschet, Aligote,  
20 Alvarelhao, Aramon, Baco blanc (22A), Burger, Cabernet franc, Cabernet, Sauvignon, Calzin, Carignane, Charbono, Chardonnay, Chasselas dore, Chenin blanc, Clairette blanche, Early Burgundy, Emerald Riesling, Feher Szagos, Fernao Pires, Flora, French Lombard, Fresia, Furmint, Gamay, Gewurztraminer, Grand noir, Gray Riesling, Green Hungarian, Green Veltliner, Grenache, Grillo, Helena, Inzolia,  
25 Lagrein, Lambrusco de Salamino, Malbec, Malvasia bianca, Mataro, Melon, Merlot, Meunier, Mission, Montua de Pilas, Muscadelle du Bordelais, Muscat blanc, Muscat Ottonel, Muscat Saint-Vallier, Nebbiolo, Nebbiolo fino, Nebbiolo Lampia, Orange Muscat, Palomino, Pedro Ximenes, Petit Bouschet, Petite Sirah, Peverella, Pinot noir, Pinot Saint-George, Primitivo di Gioia, Red Veltliner, Refosco, Rkatsiteli, Royalty,  
30 Rubired, Ruby Cabernet, Saint-Emilion, Saint Macaire, Salvador, Sangiovese, Sauvignon blanc, Sauvignon gris, Sauvignon vert, Scarlet, Seibel 5279, Seibel 9110, Seibel 13053, Semillon, Servant, Shiraz, Souzao, Sultana Crimson, Sylvaner, Tannat,

Teroldico, Tinta Madeira, Tinto cao, Touriga, Traminer, Trebbiano Toscano, Troussseau, Valdepenas, Viognier, Walschriesling, White Riesling, and Zinfandel. Rootstock cultivars which can be protected include Couderc 1202, Couderc 1613, Couderc 1616, Couderc 3309, Dog Ridge, Foex 33 EM, Freedom, Ganzin 1 (A x R #1), Harmony, Kober 5BB, LN33, Millardet & de Grasset 41B, Millardet & de Grasset 420A, Millardet & de Grasset 101-14, Oppenheim 4 (SO4), Paulsen 775, Paulsen 1045, Paulsen 1103, Richter 99, Richter 110, Riparia Gloire, Ruggeri 225, Saint-George, Salt Creek, Teleki 5A, *Vitis rupestris* Constantia, *Vitis californica*, and *Vitis girdiana*.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedures. Alternatively, transgenic seeds or propagules (e.g., scion or rootstock cultivars) are recovered from the transgenic plants. The seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants. The transgenic plants are propagated from the planted transgenic seeds under conditions effective to impart oomycete resistance to plants.

## EXAMPLES

The following examples are provided to illustrate embodiments of the present invention, but they are by no means intended to limit its scope.

### **Example 1 - Construction of Chimeric Gene**

#### **25 Cloning of *gst1* promoter**

The *gst1* promoter region from nucleotides (539 to +48) (Martini et al., "Promoter Sequences of a Potato Pathogenesis-related Gene Mediate Transcriptional Activation Selectively upon Fungal Infection," *Mol. Gen. Genet.* 236 (2-3):179-86 (1993), which is hereby incorporated by reference), was PCR amplified using DNA from potato cultivar Atlantic, using a forward primer containing a BamHI site (SEQ. ID. No. 18) as follows:

tgacggatcc taggaagttt cactttgggt gg

32

a reverse primer containing an EcoRI site (SEQ. ID. No. 19) as follows:

5 tagcgaaattc tatgtgtgggt tggtctccct tg

32

and PrimeZyme DNA polymerase (Whatman Biometra, Goettingen, Germany). The DNA was ligated into the LITMUS 38 vector (New England Biolabs, Beverly, MA) and three clones were sequenced on an ABI 377 sequencer at the Cornell

10 BioResource Center. Each clone had two to three nucleotide changes when compared to the published sequence (Martini et al., "Promoter Sequences of a Potato Pathogenesis-related Gene Mediate Transcriptional Activation Selectively upon Fungal Infection," *Mol. Gen. Genet.* 236: (2-3) 179-86 (1993), which is hereby incorporated by reference). The changes were most likely due to mistakes made by  
15 the polymerase because the promoter is extremely A-T rich and all but one of the changes were in different places in the three clones. One clone, pCPP1308, with a single change in the cis-acting region identified by Martini et al. ("Promoter Sequences of a Potato Pathogenesis-related Gene Mediate Transcriptional Activation Selectively upon Fungal Infection," *Mol. Gen. Genet.* 236: (2-3) 179-86 (1993),  
20 which is hereby incorporated by reference) was used as the source of the *gstl* promoter in all subsequent constructions.

#### Plant Transformation Constructs

The *gstl:uidA* construct was made by ligating the *gstl* promoter from  
25 pCPP1308 into pBI101 (Clontech Labs, Palo Alto, CA). For the *gstl:hrpN* and *gstl:signal sequence:hrpN* constructs (described below), the *gstl* promoter region was engineered to have a 5' HindIII site and a 3' XbaI site by the polymerase chain reaction (PCR) using pCPP1308 as the template. The forward primer had the nucleotide sequence of SEQ. ID. No. 18 and the reverse primer had a nucleotide  
30 sequence according to SEQ. ID. No. 20 as follows:

tacgtctaga tatgtgtgggt tggtctccct tg

32

For *gstI:hrpN* constructs, the *hrpN* gene of *Erwinia amylovora* (i.e., encoding a hypersensitive response elicitor identified as harpin<sub>Ea</sub>) was engineered to have a 5' XbaI restriction site and a 3' SstI restriction site by PCR using pCPP1084 (Wei et al., "Harpin, Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia Amylovora*," *Science* 257:85-88 (1992), which is hereby incorporated by reference) as the template. The forward primer had a nucleotide sequence corresponding to SEQ. ID. No. 21 as follows:

atactctaga accatgggtc tgaataacaag tggg

34

10

and the reverse primer had a nucleotide sequence corresponding to SEQ. ID. No. 22 as follows:

tcatgagctc ttaagccggc ccagcttgcc aagtg

35

15

For *gstI:signal sequence:hrpN*, the *hrpN* gene was engineered to have a BamHI site on each end. The forward primer had a nucleotide sequence corresponding to SEQ. ID. No. 23 as follows:

20

tagaggatcc ctgaatacaa gtgggctggg agcg

34

and the reverse primer had a nucleotide sequence corresponding to SEQ. ID. No. 24 as follows:

25

tcatggatcc ttaagccgcg cccagcttgc caagtg

36

The nopaline synthase terminator was extracted from pBI101 by digesting with SstI and EcoRI.

30

The nucleic acid molecule encoding the PR1-b signal sequence (of SEQ. ID. No. 11) was engineered to have XbaI restriction sites on both ends. The forward primer had a nucleotide sequence corresponding to SEQ. ID. No. 25 as follows:

atactctaga ccatgggatt ttttctcttt tca

33

and the reverse primer had a nucleotide sequence corresponding to SEQ. ID. No. 26  
as follows:

5

aggtagatcg ttttggccat gagaagagtg

30

The fragment was amplified using pSKG55 as a template (Gopalan et al., "Expression  
of the Pseudomonas Syringae Avirulence Protein AvrB in Plant Cells Alleviates its

- 10 Dependence on the Hypersensitive Response and Pathogenicity (Hrp) Secretion  
System in Eliciting Genotype-Specific Hypersensitive Cell Death." Plant Cell  
8:1095-1105 (1996), which is hereby incorporated by reference).

PrimeZyme DNA polymerase (Whatman Biometra, Goettingen,  
Germany) was used with a hot start procedure for amplification of all fragments. The  
15 amplified fragments were purified, digested with the appropriate enzymes, and ligated  
into the binary vector pPZP221 (Hajdukiewicz et al., "The Small Versatile pPZP  
Family of Agrobacterium Binary Vectors for Plant Transformation," Plant Mol. Bio.  
25:989-994 (1994), which is hereby incorporated by reference) or intermediate  
constructs, to build up the final constructs. The proper construction of pCPP1294  
20 (Figure 1) was confirmed by sequencing on an ABI 377 automated sequencer.

The final constructs were transformed into *Agrobacterium tumefaciens*  
strain GV3101 (Martin et al., "The GUS Reporter System as a Tool to Study Plant  
Gene Expression," in Gallagher, ed., GUS Protocols: Using the GUS Gene as a  
Reporter of Gene Expression, Academic Press, pp. 23-43 (1992), which is hereby  
25 incorporated by reference) by electroporation using a Bio-Rad GenePulser (Bio-Rad  
Ltd., York, UK).

**Example 2 - Inoculation with *Peronospora parasitica* Activates *gst1*  
Transcription in *Arabidopsis***

30

To evaluate the activity of the *gst1* promoter in a plant other than  
potato, transgenic *Arabidopsis* were constructed containing the *E. coli uidA* gene for  
 $\beta$ -glucuronidase (GUS) under control of the *gst1* promoter. Histochemical GUS

assays of were performed essentially as described by Martin et al., "The GUS Reporter System as a Tool to Study Plant Gene Expression," in Gallagher, ed., GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, Academic Press, pp. 23-43 (1992), which is hereby incorporated by reference. Uninoculated and

5 inoculated whole small *Arabidopsis* plants were submerged for 30 minutes on ice in six well microtiter plates in a solution of 1.5% freshly prepared paraformaldehyde in 100 mM sodium phosphate buffer, pH 7.2, containing 0.1% Triton X-100. The plants were washed twice for 5 minutes with sodium phosphate buffer pH 7.2. The plants were then submerged in a solution of 2 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-  
10 glucuronide), 50 mM sodium phosphate, pH 7.2, 0.5% Triton X-100. The solution was vacuum infiltrated into the plants and the plants were then incubated for 16 hours in the dark at 37°C. The staining was stopped by rinsing the plants several times in water and the tissue was then cleared by incubating in several changes of 70% ethanol.

15 Twenty lines were evaluated for GUS expression in uninoculated leaves, leaves inoculated with *Peronospora parasitica* isolate NOCO, and whole plants using a histochemical staining procedure (Martin et al., "The GUS Reporter System as a Tool to Study Plant Gene Expression," in Gallagher, ed., GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, Academic Press, pp 23-43 (1992), which is hereby incorporated by reference). Five lines showed more intense staining of the inoculated areas than the uninoculated areas and two lines showed no visible staining of any plant parts except the inoculated leaves (Figure 2). These results are consistent with those reported for potato and reveal that the *gst1* promoter is pathogen inducible in *Arabidopsis*. No induction of GUS activity was detected in  
20 the five lines that responded to *P. parasitica* when inoculated with *Pseudomonas syringae* pv. tomato strain DC3000, even after disease symptoms appeared (results not shown). Previously, it was reported that the *gst1* gene is induced in response to fungi, viruses, and nematodes (Strittmatter et al., "Infections with Various Types of Organisms Stimulate Transcription From a Short Promoter Fragment of the Potato  
25 *gst1* Gene," Mol. Plant-Microbe Interact. 9:68-73 (1996), which is hereby incorporated by reference), but results with bacterial pathogens were not reported.  
30

**Example 3 - Pathogen Inducible Expression of *hrpN* in Transgenic *Arabidopsis***

To generate transgenic Arabidopsis expressing *hrpN* in a pathogen-inducible manner, plant transformation vectors, pCPP1292 for cytoplasmic localization of HrpN in plants, and pCPP1294 for extracellular localization of HrpN, were constructed. (Figures 3A and 3B). *Arabidopsis* ecotype Columbia (Col-0) was transformed with the two constructs. *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in a growth chamber at 22° C and a 17 hour photoperiod. Plants with primary fluorescence 5-15 cm tall were transformed via a known vacuum infiltration method (protocol available on the Internet at <http://www.bch.msu.edu/pamgreen/vac.htm>, which is hereby incorporated by reference) adapted from Bechtold et al., *C. R. Acad. Sci. Paris* 316:1194-1199 (1993), and Bent et al., *Science* 265:1856-1860 (1994), which are hereby incorporated by reference. Seeds were collected from each plant individually, sterilized and spread on selection plates containing 150 mg/l gentamycin, 0.2 g/l Arabidopsis Growth Medium (Lehle Seeds), and 0.7% Phytagar (Gibco BRL, Bethesda, MD). Plates were vernalized for 2 days at 4°C and then moved to a growth chamber maintained at 22° C and 14 hours light. Gentamycin resistant plants were selected after 2 weeks and individual plants were transplanted to soil. Each individual T1 seedling was brought up by single seed descent and individual plant lines were selected for lack of segregation of gentamycin resistance in the T3 generation. Insertion of T-DNA was confirmed by PCR and Southern analysis.

Transgenic *Arabidopsis* lines were inoculated 2 weeks after sowing with a  $5 \times 10^4$  conidiospore suspension of *P. parasitica* isolate NOCO. Flats were covered with a humidity dome and moved to the growth chamber maintained at 18° C, 16 hours light, and 100% humidity. Plants were scored for infection 7 days after inoculation with a disease rating system adapted from Cao et al., "Generation of Broad-Spectrum Disease Resistance by Overexpression of an Essential Regulatory Gene in Systemic Acquired Resistance," *Proc. Natl. Acad. Sci. USA* 95:6531-6536 (1998), which is hereby incorporated by reference. A rating of 1, 0 conidiophores present; 2, 0-5 conidiophores present; 3, 6-20 conidiophores on a few leaves; 4, 6-20 conidiophores on all leaves; 5, 20 or more conidiophores present on all leaves. Inoculated leaves were stained with lactophenol-trypan blue (Keogh et al.,

“Comparison of Histological and Physiological Responses to Phakopsora Pachyrhizi in Resistant and Susceptible Soybean,” Trans. Br. Mycol. Soc. 74:329-333 (1980), which is hereby incorporated by reference) to observe the extent of fungal colonization under the microscope.

5 Plants were selected that lacked segregation of antibiotic resistance in the T3 generation. Lines containing the *gst1:hrpN* construct (“GN lines”) lines were tested for resistance to *P. parasitica* isolate NOCO in an initial screen.

10 Thirty lines containing the *gst1:signal sequence:hrpN* construct (“GSSN lines”) were tested for resistance to *P. parasitica* isolate NOCO in an initial screen. All but one of the lines was free of any signs of the oomycete ten days after inoculation. Ten GSSN lines were chosen for further study and inoculated by spraying with a conidiospore suspension ( $5 \times 10^4$  spores/ml) of *P. parasitica* NOCO. Northern analysis revealed that expression of *hrpN* was induced by *P. parasitica* 2 days after inoculation with strong induction at 4 days (Figure 3A). A range of 15 expression levels were observed among the ten lines, line GSSN 8-4 was chosen for further study as it displayed the highest level of expression. Production of the harpin<sub>Ea</sub> protein in inoculated plants was confirmed by immuno-blot analysis.

20 RNA was isolated from inoculated plants over a 4 day interval to analyze *hrpN* gene expression. RNA was isolated from 1g of plant tissue as described by Carpenter et al., “Preparation of RNA, in Arabidopsis Protocols,” (Martinez-Zapater, JM. and Salinas, J., eds.), Humana Press, Totowa, New Jersey, pp. 85-89 (1998). Twenty micro-gram samples were separated by formaldehyde-agarose gel electrophoresis and blotted onto Hybond N+ membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Hybridizations and 25 washing were performed according to Church et al., “Genomic Sequencing,” Proc. Natl. Acad. Sci. USA 81:1991-1995 (1984), which is hereby incorporated by reference, using P<sup>32</sup> labeled *hrpN* DNA as a probe.

The *Arabidopsis* lines GSSN 8-4 (test), Col-0 WT (wild type, control), and Col-0 EV (empty vector, control) were inoculated by drop inoculation with a 30 conidiospore suspension ( $5 \times 10^4$  spores/ml) of *P. parasitica*. Plants were maintained in a growth chamber (16 hours of light, 18° C, 100% humidity) and were scored for infection ten days post inoculation. Nearly all (29 out of 30) 8-4 plants were free of

any signs of *P. parasitica* (Figure 4A). Trypan blue staining showed that growth of the oomycete was strongly inhibited in GSN 8-4 plants. Extensive hyphal growth was evident in Col-0 WT and Col-0 EV plants (Figure 4B).

Plants were rated for disease severity based on the number of  
5 conidiophores per leaf. Nearly all GSN 8-4 plants received a disease rating of 1 with  
only one being scored 3. The majority of the Col-0 WT and Col-0 EV plants were  
rated 5, the remainder were rated 4 (Figure 5).

This example demonstrates that pathogen inducible expression of the  
harpin<sub>Ea</sub> hypersensitive response elicitor of *Erwinia amylovora* in transgenic plants is  
10 a potentially useful strategy for engineering plants for disease resistance. Challenge  
with *Peronospora parasitica* resulted in accumulation of *hrpN* mRNA, production of  
harpin<sub>Ea</sub> protein, and resistance to *P. parasitica*. Upon challenge by *P. parasitica*, it is  
believed that the transgenic plants most likely mount a hypersensitive response at the  
site of inoculation, conferring resistance. Subsequently the plants may develop  
15 systemic resistance.

For the purposes of the present invention, the *gst1* promoter was most applicable to the *Arabidopsis/P. parasitica* pathosystem since it is well documented that transcription from *gst1* is activated by other oomycete pathogens (Martini et al., “Promoter Sequences of a Potato Pathogenesis-related Gene Mediate Transcriptional Activation Selectively upon Fungal Infection,” *Mol. Gen. Genet.* 236: (2-3) 179-86 (1993), which is hereby incorporated by reference). Additionally, it has been reported that *gst1* activation is stimulated by ascomycete, viral, and nematode infection and mycorrhization (Strittmatter et al., “Infections with Various Types of Organisms Stimulate Transcription From a Short Promoter Fragment of the Potato *gst1* Gene,” 20 *Mol. Plant-Microbe Interact.* 9:68-73 (1996), which is hereby incorporated by reference). Therefore, it is possible that both *gst1:hrpN* and *gst1:signal sequence:hrpN* constructs may also confer resistance against ascomycete, virus, and nematode infection, as well as mycorrhization.

Although the invention has been described in detail for the purpose of  
30 illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

All of the references designated as being incorporated herein by reference are intended to be incorporated in their entirety unless specific portions thereof have been identified with particularity.